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Patent- og Varemærkestyrelsen
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31 March 2005

Pia Høybye-Olsen

PATENT- OG VAREMÆRKESTYRELSEN

**Title:** RAPID EXTRACTION OF BIOCHEMICAL AND GENOMIC MATERIAL FROM ENDOSPORES OF GRAM-POSITIVE BACTERIA BY MEANS OF HIGH FREQUENCY ALTERNATING FIELDS

**Prior art:**

Mainelis et al., (2002) Effect of electrical charges and fields on injury and viability of airborne bacteria. Biotechnology and bioengineering, 79 (2): 229-241

Spilimbergo et al., (2003) Inactivation of Bacteria and Spores by Pulse Electric Field and High Pressure $CO_2$ at Low Temperature. Biotechnology and bioengineering, 82 (1): 118-125

- *B. cereus* start CFU:5 × $10^6$, number of pulses: 20, E=25 kV/cm, decrease (log(No/N)=0.5

Grahl and Märkl (1996) Killing of microorganisms by pulsed electric fields. Appl. Microbiol. Biotechnol. 45:148–157

- A high-voltage generator with 5-15 kV d.c. voltage and a pulse frequency of 1-22 Hz.
- The maximum capacity was 5.0 µF.

Cho et al., (1999) Kinetics of Inactivation of *Bacillus subtilis* Spores by Continuous or Intermittent Ohmic and Conventional Heating biotechnology and bioengineering, 62(3): 368-372,

Cserhalmia et al., (2002) Inactivation of *Saccharomyces cerevisiae* and *Bacillus cereus* by pulsed electric fields technology. Innovative Food Science & Emerging Technologies 3:41-45

- *B. cereus* cells were less sensitive to PEF treatment. The reduction in microbial count of *B. cereus* cells was hardly more than one log cycle using 10.4 pulses at 20 kV/cm.

Lado and Yousef (2002). Alternative food-preservation technologies: efficacy and mechanisms. Microbes and Infection 4:433–440

- Pulsed electric field (PEF) treatment is based on the delivery of pulses at high electric field intensity (5-55 kV/cm) for a few milliseconds.

**ABSTRACT**

The present invention describes a fast and simple method for the lysis of eukaryotic and prokaryotic cells and spores. The invention is aimed mainly for use in combination with detection devices, making it possible to dramatically speed up the detection of e.g. pathogenic micro-organisms by releasing intracellular components that can be detected or purified by other means, e.g., released genomic DNA can be used as substrate in a PCR reaction.

**FIELD OF THE INVENTION**

The present invention describes a method for rapid extraction of biochemical and genomic material from endospores of Gram-positive bacteria in particular spores from the *Bacillus cereus* group utilizing electrolysis. More specifically, the invention relates to the use of alternating electric fields for the rupturing of cell and spore membranes.

**BACKGROUND OF THE INVENTION**

In response to Bioterrorism threats it has become increasingly important to perform rapid and precise detection of biological warfare agents. The *Bacillus anthracis* bacterium is a member of the endospore forming *Bacillus cereus* group. *Bacillus anthracis* is a highly lethal biological warfare agent that is easy to obtain, store, and apply as a bioweapon. In order to make a reliable detection of *Bacillus anthracis* the DNA must be analyzed, since phenotypic differences between the members of the *Bacillus cereus* group in some instances are is less than 1%.

As an example, *Bacillus cereus* differs only from *Bacillus anthracis* because the latter contains two additional plasmids called pXO1 and pXO2. Avirulent strains of *Bacillus anthracis* lacking pXO1 and pXO2 are virtually indistinguishable from *Bacillus cereus*. Theoretically, transfer of the PX01 and PX02 plasmids into members of the *Bacillus cereus* group will turn these bacteria into functional *Bacillus anthracis*. For this reason DNA analysis and discrimination of the plasmids pXO1 and pXO2 by means of DNA hybridization, sequencing, or PCR is the only valid method for determining if the detected organism is *Bacillus anthracis*.

Due to their capability to resist harsh environments, the liberation and extraction of DNA from an endospore is a difficult task. The normal procedure used in the detection of *Bacillus anthracis* is to germinate the spores in a culture substrate, collect the bacteria and subsequently extract the DNA from the vegetative bacteria, a procedure that can take many hours up to a day (ref). Other methods include elaborate techniques like mechanical disruption, freeze/thaw cycles or chemical treatment (Johns et al 1994). However, the spore coat and cortex are biochemical structures developed for long term hibernation that can last thousands of years. A famous example is a bacterium revived from an endospore found in the guts of bee embedded in amber (Cano & Borucki 1995). Furthermore, mechanical disruption (bead beating) results in poor quality of the released DNA (Levi et al 2003). Using present technology, it is possible to release DNA within 5-10 minutes from endospores by combing physical, mechanical, and chemical treatment, but even 5 minutes for DNA

extraction is considered long time when the application is a monitoring of bioterrorism attacks carried by aerosols. The use of elaborate multistep procedures is not optimal in the stressful situation that a possible anthrax attack is. For this reason there is a need for a technology that allows rapid (within seconds) hands-off single step DNA extraction from endospores of Gram positive bacteria.

Gram positive bacteria of the genus *Bacillus* and *Clostridia* are capable of undergoing a process at the end of the exponential growth phase called sporulation. During sporulation the bacteria form a rugged spore that is capable of resisting harsh environments. The spore is a dormant structure with only a few metabolic active enzymes that induced germination when the spore is exposed to nutrients. The spore is very different in it biochemical composition as seen from table 1 and also subtle physiological differences exist (see Table 2.)

**Table 1**. Biochemical compositional differences between spores and vegetative cells of *Bacillus* species.

| | Levels of molecules (µmol/g [dry weight] | |
|---|---|---|
| Small molecule | *Bacillus* spore | *Bacillus* vegetative cell |
| NADH | <0.002 | 1.95 |
| NAD | 0.11 | 0.35 |
| NADPH | <0.001 | 0.52 |
| NADP | <0.018 | 0.44 |
| ATP | <0.005 | 3.6 |
| ADP | 0.2 | 1 |
| AMP | 1.2-1.3 | 1 |
| 3PGA | 5-18 | <0.2 |
| DPA | 410-470 | <0.1 |
| $Ca^{2+}$ | 380-916 | |
| $Mg^{2+}$ | 86-120 | |
| $Mn^{2+}$ | 27-56 | |
| $H^{+}$ | 6.3-6.5 | 7.5-8.2 |

The biochemical structures and the dormant physiological state makes the endospore an extremely mechanical, chemical, and heat resistant entity that poses a particular problem in terms of rapid sample preparation and DNA extraction of biological warfare agents for rapid

identification. The spores can resist e.g. prolonged boiling without breaking apart. The environmental fate of the spore is not known in detail. The spores will survive 'indefinitely' in dry and protected environments. Excavations in Kruger National Park in South Africa revealed *B. anthracis* spores that were more the 200 years old (dated by the 14C method) and able to germinate in the laboratory. For an overview of an endospore see Figure 1.

We speculated, that since electrical rupture of cellular membranes is a widely used discipline arising from electrophysiology where it is accepted and implemented as a standard protocol for gaining electrochemical access to the intracellular compartment of the cell under investigation, then it should be possible to apply this technology or modifications of it to extract DNA from endospores.

**Table 2.** Physiological differences between spores and vegetative cells from *Bacillus* species.

| Vegetative cells | Endospores from Gram positive bacteria |
|---|---|
| 4 g water/ g [dry weight] | 1.5 g water/g [dry weight] (in core)<br>4 g water/g [dry weight] (outer spore) |
| Cell membrane and cell wall | Spore coat, cortex and nucleoid |
| $Ca^{2+}$ buffered in solution | Very high intracellular $Ca^{2+}$ concentration bound to dipicolinic-acid that works as a chelating agent. (390-916 µmol/g [dry weight]. Also elevated $Mg^{2+}$ and $Mn^{2+}$ |
| Intracellular pH 7 | Intracellular pH 6 |
| dipicolinic-acid almost absent | High concentration of dipicolinic-acid and 3-phosphoglyceric acid |
| Full metabolism and catabolism | Lack of ATP and pyridine nucleotides |
| Low heat and mechanical resistance and some chemical resistance | High heat, chemical and mechanical resistance |

For example, the technique is used to penetrate neuronal cells with sharp glass electrodes with tip-sizes in the range 0.05-0.1 µm. The tip is positioned at the cell membrane and the oscillating field is applied between the tip and the ground electrode. Normally an oscillating field at a frequency of 1 kHz in the 200-400 mV range with a time duration of 100-200 ms is sufficient to cause breakdown of the membrane make the large electrode, compared to the molecular structures of the cell, enter the interior of the cell. The treatment generates a

physical hole in the membrane that slowly seals around the glass electrode depending on how clean the exterior is of the glass electrode.

Another approach based on the same principles of electrical breakdown is utilized in another discipline of electrophysiology know as patch-clamp. The glass electrode is created with a large smooth tip (5-10 μm wide), which can be positioned onto the surface without causing mechanical damage. The interior of the electrode forms an opening of 0.5-2 μm which contacts the cellular membrane when the electrode is pressed against the cell. Large pressures can be achieved without causing any damage, which illustrates how strong the cellular membrane is. However, a short electrical pulse of 10-20 ms with a potential in the range 500-1000 mV and oscillations in the range of 1-2 kHz causes a gentle rupture of the cellular membrane with immediately resealing onto the glass surfaces of the electrode.

Electrical fields have also been used to cause temporal breakdown of cellular membrane facilitating transfer of large molecules like DNA plasmids into the cells. This treatment is done in bulk solutions with no close electrical contact between the electrodes and the cells. Thus larger fields are required but the principle is widely applied in cloning of genes. The exact same methodology is utilized to kill bacteria in the food industry by applying longer time exposure and properly causing major macromolecules to leak out from the cells.

Pulsed electric field (PEF) treatment is an established food-preservation technology based on the delivery of pulses at high electric field intensity (5-55 kV/cm) for a few milliseconds. The theoretical base of PEF technology, typically suitable for liquid foods, is the effect of an external electric field on the cell membrane. This effect appears in the breakdown of lipid bilayers and biological cell membranes. The breakdown can be explained with the following processes

- dielectric breakdown
- threshold transmembrane potential compression of cell membrane
- visco-elastic properties of the cell membrane
- fluid mosaic arrangement of lipids and proteins in cell membrane
- structural defects in the cell membrane

It is therefore possible to create a microstructure that would facilitate the electrolysis of cellular membranes using the strategy above and explore this structure for its ability to cause DNA to leak out of endospores either by direct pore forming mechanisms by concomitant disruptions of the spore's integrity.

The issue of the amount of applied electric potential actually appearing across the solution is particularly important in microstructures using a two electrode configuration for applying an electroporating voltage to the cell or spore solution, i.e., the voltage drop at the electrode-solution interface is significant in comparison with the applied voltage. The solution in which the experiments are performed sets limits to the potential gradient over time. E.g., a small applied voltage (<2V) gives a very small potential gradient across the electrodes even at long pulse times (>100 µs) - a peak shaped gradient is created. The maximum magnitude of applied voltage in solution can be calculated by convoluting exponential functions for the instrument rise time and charging time. For example, an instrument rise time of 8 µsec and a charging time in the range 20-40 µs give a maximum of 15-18% of the applied amplitude. The maximum is achieved approximately 10-15 µs after application of the pulse. After 50-75 µs the potential drop is effectively zero because all the applied voltage appears at the electrode-solution interface. At higher voltages the interfacial impedances becomes negligible and the current that passes between the electrodes is limited by the solution conductance.

Limited data is available on the damage to DNA resulting from exposure to electrical fields. Some reports on DNA damage rely on the calculation of strand scission factor (SSF) as a measurement for DNA damage. However, the SSF is only applicable to eukaryotic cells and is only measuring the release of DNA from the nucleus of the cells, but not actually determining the quality of the released DNA.

Chemical modification might occur due to the formation of radicals at the electrodes. These radicals will rapidly react with each other, or with the DNA molecule. Frequencies in the order of 1 - 100 kHz mean that polarity switches quickly every 1 ms to every 10 µs. During this time-span radicals have hardly any time to diffuse far away from the electrode by means of Brownian motion, and most likely, will react with the radical generated during the oppositely charged electrode cycle. Furthermore, these extremely short cycle times will not be enough to move the DNA molecules towards an electrode fast enough to have them collect at the electrode surface, before the next cycle will try to electro-migrate it towards to other electrode. This means that the majority of the DNA molecules will not get into contact with either of the electrodes.

Furthermore, it is known from DNA migration studies, that the DNA molecule is aligned along the electrical field prior to movement, again arguing for the immobility of the DNA.

## SUMMARY OF THE INVENTION

Here we describe a method and a microstructure facilitating the method for extracting DNA from endospores of bacteria from the *Bacillus* group.

The purpose of the present invention is to perform a rapid DNA extraction from highly mechanical, chemical, and heat resistant endospores of Gram-positive bacteria, including *Bacillus anthracis*, to enable rapid DNA detection.

This purpose is obtained by a method and a structure comprising the combined usage of a pulsed electrical field induced over the spores, said usage consists of a fluidic structure containing a solution of spores embedded between or adjacent to an electrical field that can be varied in frequency and amplitude and applied for a variable time, and a set of optimal parameter settings for the said usage.

The present invention describes a method that enables the application of a pulsed electrical field across a solution containing spores. As a result of said method DNA is released within a period of 5 seconds after initialization of said method.

In its first embodiment, this invention provides a method for rapid extraction of DNA from spores utilizing a two electrode configuration with an intercalated solution containing endospores. The electrodes are connected to a voltage source and frequency generator allowing a pulsed electrical field to be induced on the solution.

In its second embodiment, the invention utilizes a micro-fluidic device that performs the same functions as above and might be an integrated part of a sample preparation system for rapid DNA extraction.

The essence of the method is to introduce pore forming in endospores or cells by a pulsed electrical field causing leakage of macromolecules as e.g. DNA into the surrounding solution.

In yet another aspect of the invention, the quality of the released DNA as a consequence of the application of said method is not affected as measured by subsequently running PCR or visually inspecting the quality of DNA (after agarose gel electrophoresis and staining).

**DETAILED DESCRIPTION OF THE INVENTION**

In this section we provide a detailed description of the invention and its application in extracting DNA from endospores of bacteria from the *Bacillus* group. The mechanism of action is the generating pores in the spores as it is generally known from electrophysiological and widely described by (Lee & Kolodney 1987;Tsong 1991;Chang et al 1992;Tsong & Su 1999).

The voltage across a cell membrane of a spherical cell at different locations in a homogeneous electric field for a duration t, can be calculated from:

$$V_m = 1.5 r_c \cos\alpha \left[ 1 - e^{\frac{-t}{\tau_m}} \right]$$

Where E is the electric field strength, $r_c$ is the radius of the cell, $\alpha$ is the angle in relation to the direction of the electric field, and

$$\tau_m = r_c C_m ((R_{int} + R_{ext})/2$$

gives the membrane relaxation time. $C_m$ is the membrane capacitance and $R_{int}$ and $R_{ext}$ are the specific resistivities of the intracellular and extracellular media.

The applied electric field will generate large depolarizing and hyperpolarizing transmembrane potentials at the cathode- and the anode-facing poles of the cell, respectively. When the membrane reaches a critical value, typically 0.2-1.5 V for mammalian cells, dielectric membrane breakdown will occur, which results in pore formation. Pore density will follow the $V_m$-gradient and is highest at the polarized (electrode-facing) ends of the cell. During the effective pore-open time, cell constituents, solutes and macromolecules can move freely between the surrounding medium and the interior of the cell.

The conductance caused by dielectric breakdown of membranes is proportional to the amplitude and duration of the electrical field. Pores of 1 nm size have been reported for human erythrocytes and the size of the pores was determined by field strength, pulse duration, pH, and ionic strength of the suspending medium. Investigation with electron-microscopy has revealed pores in the range 6-240 nm. In charge-pulse studies on irreversible breakdown of planar lipid membranes, pores with diameters up to 400 µm have been observed.

*Voltage profile in microstructures*

The issue regarding the amount of the applied potential actually appearing across the solution is particularly important in microstructures using a two electrode configuration for applying an electroporating voltage to the cell or spore solution. Due to this, the voltage drop at the electrode-solution interface is significant in comparison with the applied voltage.

To persons skilled in the art, it is well known that the solution used in the experiment sets limits on the potential gradient over time. E.g., a small applied voltage (<2 V) results in a very small potential gradient across the electrodes, even at long pulse times (>100 µs) generating a peak shaped gradient. The maximum magnitude of applied voltage in solution can be calculated by convoluting the exponential functions for the instrument rise time and charging time. For example, an instrument rise time of 8 µs and a charging time in the range 20-40 µs give a maximum of 15-18% of the applied amplitude. The maximum is achieved approximately 10-15 µs after application of the pulse. After 50-75 µs the potential drop is effectively zero because all the applied voltage appears at the electrode-solution interface. At higher voltages the interfacial impedances becomes negligible and the current that passes between the electrodes is limited by the solution conductance.

The resistance of a 100 mM KCl solution for distances greater than 10 µm is generally found in the range of 30 ± 3 kΩ.

*Spore coat*

The spore coat consists of protein that gives mechanical protection to the entire spore structure. *Bacillus cereus* has a spore coat that consists of one protein whereas *Bacillus subtilis* has more than 20 proteins have been identified in the coat. Furthermore, the coat is a functional molecular sieve protecting the spore interior against all but the smallest molecules, although the spore coat has be associated with resistance to halogens as chloride and iodine. The coat also participates in germination; it has been shown that spores with mutations in the spore coat also are the most defective in regard of germination.

*Spore cortex*

In the vegetative cell of *Bacillus* species the cell wall peptidoglycan is composed of repeating disaccharides of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) in ß-1,4 linkage (see figure below). Peptide chains (initially L-alanine-D-glutamate-meso-diaminopimelic acid [Dpm]-D-alanine-D-alanine) are attached to the NAM residues; approximately 35% of all PG in vegetative cells participates in cross-linking. In contrast to vegetative cell PG, which can exhibit significant variations between different *Bacillus* species, the basic features of the structure of the cortical PG are similar in all species. The structure of the cortical PG differs in two major respects from that of the vegetative cell PG.

1. Approximately 50% of NAM residues in the cortex are present as muramic acid-rho-lactam (MAL) with the majority of the MAL residues spaced at every second muramic acid position in the glycan strand

2. Approximately 25% of NAM residues carry only a single L-alanine and since MAL residues do not carry a peptide side chain, only 1/4 as many Dpm residues are available to participate in cross-link formation in spore cortex compared to the growing cell. As a consequence spore PG is much less cross-linked than in the vegetative cell. However, the exact molecular arrangement in the spore cortex is not known. It has been shown that the spore cortex is involved in osmotic swelling and shrinking of the spore.

*DNA quality*

As a consequence of the applied and alternating electric potential, chemical alteration of the nucleotide bases of the DNA due to oxidation or reduction reactions at the electrodes could occur. This may possibly result in bad nucleotide recognition by the thermo-stable DNA polymerase during subsequent DNA amplification, yielding reduced amplification rates or misincorporation of nucleotides.

The invention will further be described in the following examples, which do not limit the scope of the invention described in the claims

## EXAMPLES

### *Example 1*

DNA quality following the application of an alternating electric potential

Real-time PCR analysis was performed on an Opticon DNA engine (MJ research) using the TaqMan Universal PCR Master Mix system (Applied Biosystems), which is an integrated solution containing buffer, dNTP's and *Taq* polymerase. Two primers were added (269-16-23spacer1 5'-TAT GAG CTA CAC TGT TAT CTA GTT TTC AAA GAA-3' and 270-16-23spacer2 5'-TTT CCG TGT TTC GTT TTG TTC AG-3') at a final concentration of 900 nM and a fluorescent TAQMAN probe (**FAM**-ACT TCT CTC ATA TAT AAA TGT-**MGB-NFQ**) at 100 nM all aimed at amplifying the inter-genetic spacer of the 16S and 23S tRNA genes of *Bacillus thurigiensis*. The standard PCR used a 15 µl sample volume and PCR was initiated by incubation 15 minutes at 95 °C to activate the *Taq* polymerase. Subsequently 40 cycles of a two step PCR reaction was applied being a 15 sec. melting step at 94 °C and a combined annealing and extension step of 60 sec. at 60 °C. At the end of each cycle the fluorescence was measured on-line to analyze PCR product formation.

The chromosomal DNA from two ml (approximately $10^9$ cells/ml) of an overnight culture of *Bacillus thuringiensis* subsp. *kurstaki* was isolated using the method of Boe et al. (Boe et al 1989)). The purified DNA was dissolved in 75 µl TE-buffer, meaning that a $10^{-6}$ dilution should contain DNA from approx. 27 cells / µl.

Four µl of a $10^{-6}$ dilution of the chromosomal DNA solution (equivalent to ~100 genome copies) was used to analyze the possible damaging effect of high frequency electrical fields on diluted DNA samples. The standard detection PCR was performed to probe the influence of the electrical field(s) applied. In details, 20 µl of a $10^{-6}$ dilution of *Bacillus thurigiensis* chromosomal DNA was submitted to a 10V, 100 kHz high frequency electrolysis field at 5, 10, 20, or 30 seconds. Subsequently, four µl (the equivalent of 100 copies of the chromosome) of the treated DNA was subjected to real-time PCR analysis. All samples subjected to the electrolysis conditions, were detected after 34 cycles of PCR, meaning that no loss of detection sensitivity was observed. The fact that all differently treated DNA samples are detected at the same $C_T$ point further indicates that chemical reduction or oxidation of nucleotides bases is of minor effect on the sensitivity of the detection PCR (See Figure 1). However, increasing the duration of the electrolysis procedure even further, (30, 90, or 180 seconds) shows that electrolysis time does have an effect when prolonged procedures are applied (See Figure 2). Electrolysis for more than 30 sec presumably results in the release and over-saturation of target DNA, subsequently giving poor PCR detection as measured by quantitative PCR

***Example2***

Spore lysis following the application of an alternating electric potential as measured by quantitative PCR

One hundred mg of Biobit *Bacillus thuringiensis* subsp. *kurstaki* containing $3.2 \times 10^9$ spores / g (Valent BioSciences Corp, Libertyville, USA) was resuspended in 1 ml of demineralized water and centrifuged for 90 sec. at 12000 rpm. This procedure was repeated 4 times. The supernatant was discarded. The final solution contained approximately $3.2 \times 10^8$ spores. This solution was diluted to a final concentration of $3.2 \times 10^5$ spores / ml. and subsequently used for electrolysis and PCR.

Keeping the frequency and voltage constant (100 kHz and 10 V, respectively), the duration of the electrolysis procedure was examined using variations of exposure time – ranging from 5 to 30 seconds. As evident from figure 3, there is no noticeable effect on the $C_T$ value, demonstrating that lysis of the spores is virtually time-independent. However, increasing the duration of the electrolysis procedure even further (30, 90, and 180 seconds, respectively), shows that electrolysis time does have an effect when prolonged procedures are applied (See Figure 4). Electrolysis for more than 30 sec presumably results in the release and over-saturation of target DNA, subsequently giving poor PCR detection.

***Example3***

The effects of varying frequencies (10-100 kHz) on spore electrolysis efficiency.

One hundred mg of Biobit *Bacillus thuringiensis* subsp. *kurstaki* containing $3.2 \times 10^9$ spores / g (Valent BioSciences Corp, Libertyville, USA) was resuspended in 1 ml of demineralized water and centrifuged for 90 sec. at 12000 rpm. This procedure was repeated 4 times. The supernatant was discarded. The final solution contains approximately $3.2 \times 10^8$ spores. This solution was diluted to a final concentration of $3.2 \times 10^5$ spores / ml. and subsequently used for electrolysis and PCR.

Keeping the voltage and time constant (at 10 V and 30 sec, respectively). Figure 4 shows the results of this experiment and as apparent, the high frequency of 100 kHz showed a decrease in $C_T$ (threshold cycle), thus demonstrating release of amplifiable DNA from the spores. Lowering the frequency to 50 kHz or 10 kHz did not give rise to any improvement in the $C_T$ values (i.e. a lowering of $C_T$ compared to untreated controls).

***Example 4***

Determination of the minimal effective voltage for electrolysis of spores.

Keeping the frequency and time constant (100 kHz and 30 sec, respectively), the voltage of the electrolysis procedure was examined using variations of exposure voltage – ranging from 3 to 10 Volts (3, 5, 7, and 10 V, respectively). As evident from figure 5, it is apparent that a threshold voltage value $V_T$ for membrane/ spore rupture exists. Thus, the applied voltage is a critical parameter highly dependent on the interfacial electrode resistance, e.g. a small, applied voltage (<2 V) gives a very small potential gradient across the electrodes even at long pulse times (>100 µs); a peak shaped gradient is created.

The maximum magnitude of applied voltage in solution can be calculated by convoluting exponential functions for the instrument rise time and charging time. For example, an instrument rise time of 8 µs and a charging time in the range 20-40 µs gives a maximum of 15-18 % of the applied amplitude. The maximum is achieved approximately 10-15 µs after application of the pulse. After 50-75 µs the potential drop is effectively zero because all the applied voltage appears at the electrode-solution interface. At higher voltages the interfacial impedances becomes negligible and the current that passes between the electrodes is limited by the solution conductance

**BRIEF DESCRIPTION OF THE FIGURES**

Figure 1. Increasing the duration of the electrolysis procedure keeping the frequency and voltage constant (100 kHz and 10 V, respectively) results in no noticeable effect on the $C_T$ value, demonstrating that lysis of the spores is virtually time-independent

Figure 2. Increasing the duration of the electrolysis procedure even further compared to data presented in figure 1 shows that electrolysis time does have an effect when prolonged procedures are applied. Electrolysis for more than 30 sec presumably results in the release and over-saturation of target DNA, subsequently giving poor PCR detection.

Figure 3. Identical real time PCR detection sensitivity of low copy *Bacillus thuringiensis* chromosomal DNA using varying electrolysis times (5 - 180 seconds).

Figure 4. Increasing the frequency to 100 kHz results in a downward shift in CT, demonstrating lysis of the spores at this frequency compared to both 10 kHz, 50 kHz and the untreated controls

Figure 5.. Increasing the voltage in the electrolysis procedure clearly demonstrates the existence of threshold voltage value $V_T$ for membrane/ spore rupture – the implication of this observation is further discussed in the text.

**GLOSSARY:**

As used herein, "amplifying" refers to the process of synthesizing nucleic acid molecules that are complementary to one or both strands of a template nucleic acid molecule. Amplifying a nucleic acid molecule typically includes denaturing the template nucleic acid, annealing primers to the template nucleic acid at a temperature that is below the melting temperatures of the primers, and enzymatically elongating from the primers to generate an amplification product. Amplification typically requires the presence of deoxyribonucleoside triphosphates, a thermo-stable DNA polymerase enzyme (e.g., Platinum® Taq) and an appropriate buffer and/or co-factors for optimal activity of the polymerase enzyme (e.g., $MgCl_2$ and/or KCl).

As used herein "thermostable DNA polymerase" refers to a polymerase enzyme that is heat stable, i.e., the enzyme catalyzes the formation of primer extension products complementary to a template and does not irreversibly denature when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded template nucleic acids. Thermo stable

polymerases have been isolated from *Thermus flavus, T. ruber, T. thermophilus, T. aquaticus, T. lacteus, T. rubens, Bacillus stearothermophilus* and *Methanothermus fervidus.*

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**CLAIMS:**

1. A method for making intracellular components accessible from spores by applying a high frequency alternating electrolyzing field.
2. The method described in claim 1, more precisely aimed at the extraction of DNA.
3. The method described in claim 2, more precisely aimed at purifying DNA.
4. The method described in claim 1-3, more precisely aimed for use in DNA detection.
5. The method described in claim 4 more precisely aimed for use in diagnostic analysis.
6. The method described in claim 5, more precisely aimed for use in PCR.
7. The combination of methods in claims 4 -6.
8. The method described in claim 6, more precisely aimed for use in integrated PCR devices.
9. The method described in claim 8, more precisely aimed for use in mobile PCR devices.
10. The method described in claim 9, more precisely aimed for use in biological warfare agent detection.
11. The method described in claim 1, more precisely aimed at bacterial spores.
12. The method described in claim 11, more precisely aimed at spores from Gram positive bacteria.
13. The method described in claim 12, more precisely aimed at spores from the genus *Bacillus* and *Clostridia*.
14. The method described in claim 13, more precisely aimed at spores from the *Bacillus cereus* group.
15. The method described in claim 14, more precisely aimed at spores from the species *Bacillus anthracis*.
16. The combination of the methods described in claims 1 – 10, with claims 11 – 15.
17. The method described in claim 4, more precisely aimed at detecting prokaryotic cells.
18. The methods described in claim 17, where the aim is to liberate DNA from bacterial cells.
19. The method described in claim 18, more precisely aimed at heat resistant bacterial cells.
20. The method described in claim 19, more precisely aimed at thermophilic and caldoactive bacteria.
21. The combination of the methods described in claims 1-10 and 19, more precisely aimed at the detection of heat resistant bacteria by PCR.

22. The method described in claim 19, more precisely aimed at the detection of heat lysis resistant bacteria.
23. The method described in claim 20, more precisely aimed for use with Mycobacteria.
24. The methods described in claims 1 -10, aimed for use with eukaryotic cells.
25. The method described in claim 24, more precisely aimed for use with mammalian cells.
26. The method described in claim 25, more precisely for use human cells.
27. The method described in claim 24, more precisely for use with fungal cells.
28. The method described in claim 24, more precisely for use with plant cells.
29. The methods described in claim 1-10, more precisely aimed for use with viruses.

Design claims

30. The methods described in claims 1-10, for use in a capillary design where the cell or spore suspension is contained in-between two or more electrode surfaces.
31. The design described in claim 30
32. The method described in claims 1-4, with lysis induced by the application of high frequency alternating fields.
33. The method of claim 32, more precisely with applied frequencies between 8000 and 200,000 Hz.
34. The method of claim 32, more precisely with applied pulse sequences between 1 and 60 sec.
35. The method of claim 32, more precisely with the application of short breaks in-between pulses.
36. The method of claim 32, more precisely with applied voltages between 6 and 40 V.
37. The method of claim 32, more precisely with application of the cells or spores suspended in demineralized water.
38. The method of claim 32, more precisely with application of the cells or spores suspended in PCR buffer formulation.
39. The combination of claims 32-38
40. The methods of claim 1-4, for use in combination with a capture device.
41. The method of claim 40, more precisely for use in combination with a spore capture device.
42. The method of claim 41, more precisely for use in combination with a cell capture device.
43. The method of claim 41, more precisely in combination with an electrostatic capture device.

44. The method of claim 41, more precisely as part of integrated spore capture and lysis design.
45. The combination of claims 1-10 with claims 40-44.

5

**Figure 1:**

**Efficient and highly similar PCR detection of spores after just 5 to 30 seconds of electrolysis**

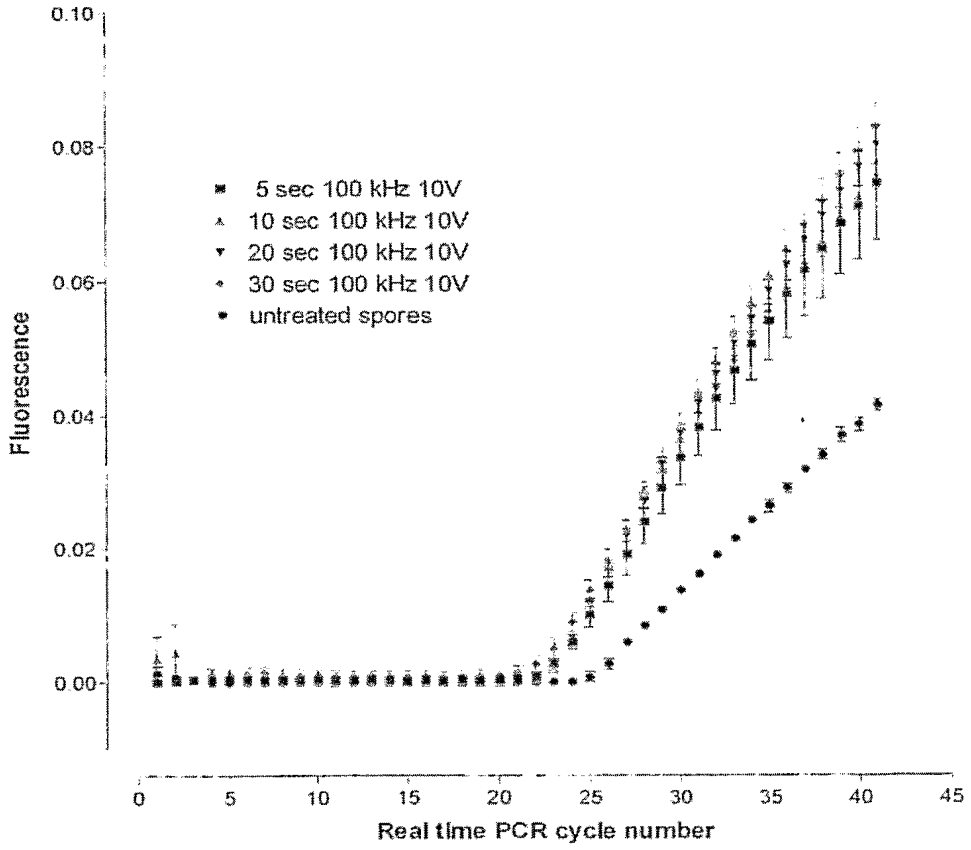


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**Figure 2:**

Prolonged electrolysis can reduce the PCR detection sensitivity, presumeably by producing excess amounts of template DNA.

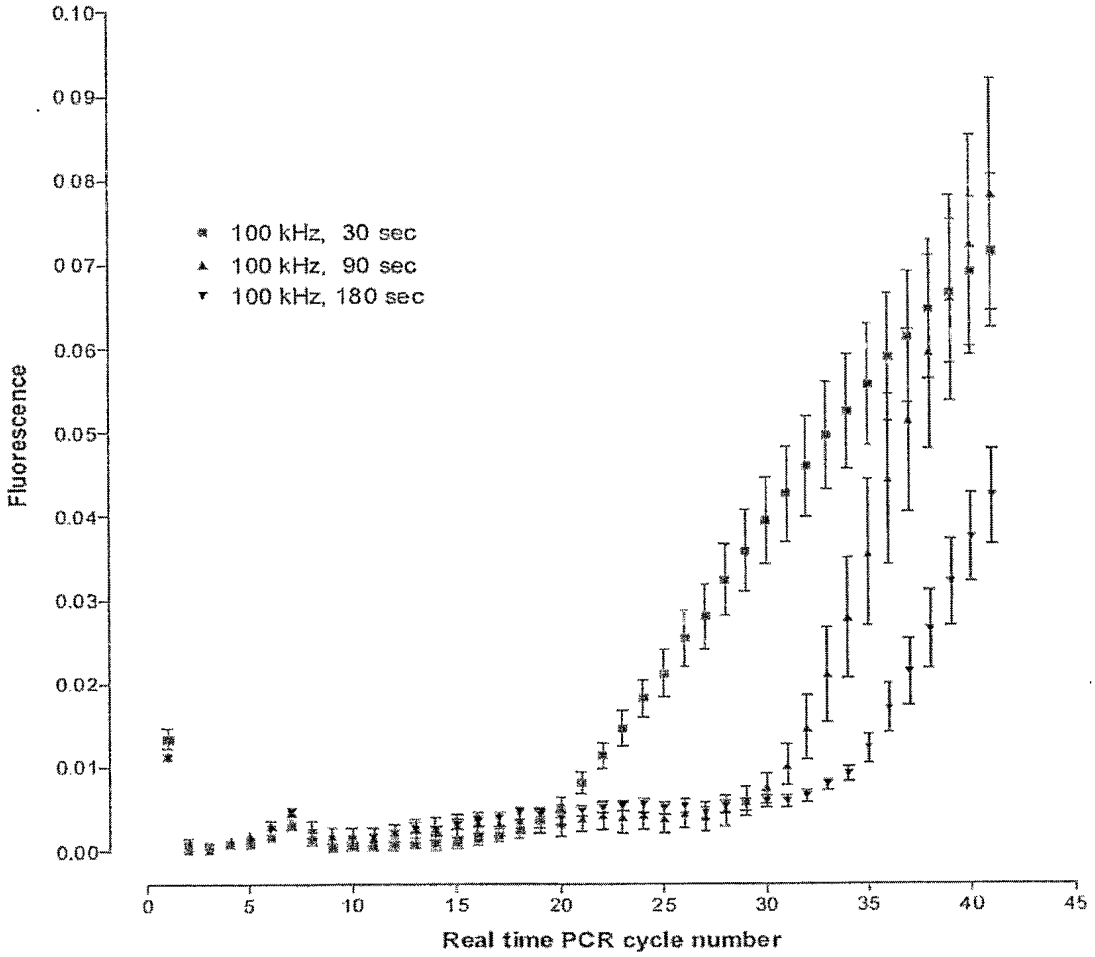

**Figure 3:**

Longer electrolysis timespans have no adverse effects on the quality of target DNA as measured by PCR sensitivity.

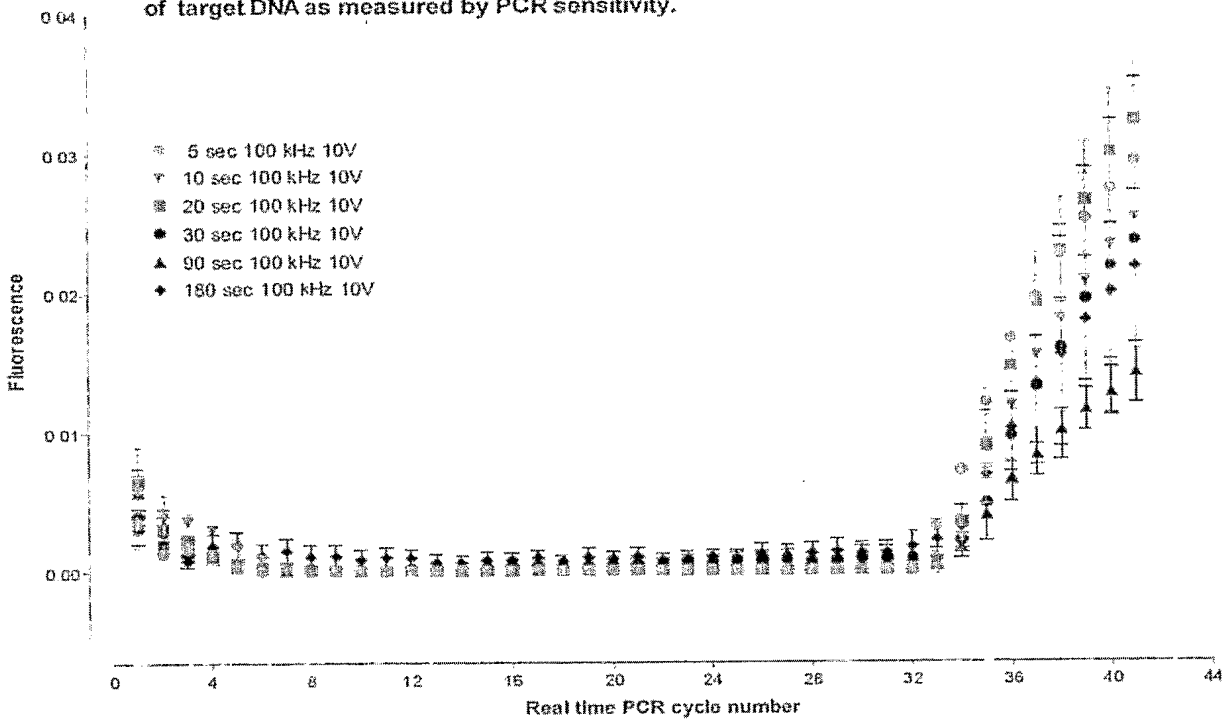

**Figure 4**

**Electrolysis of spores using either 10 or 50 kHz does not influence DNA yield, whereas 100 kHz results in a significant effect.**

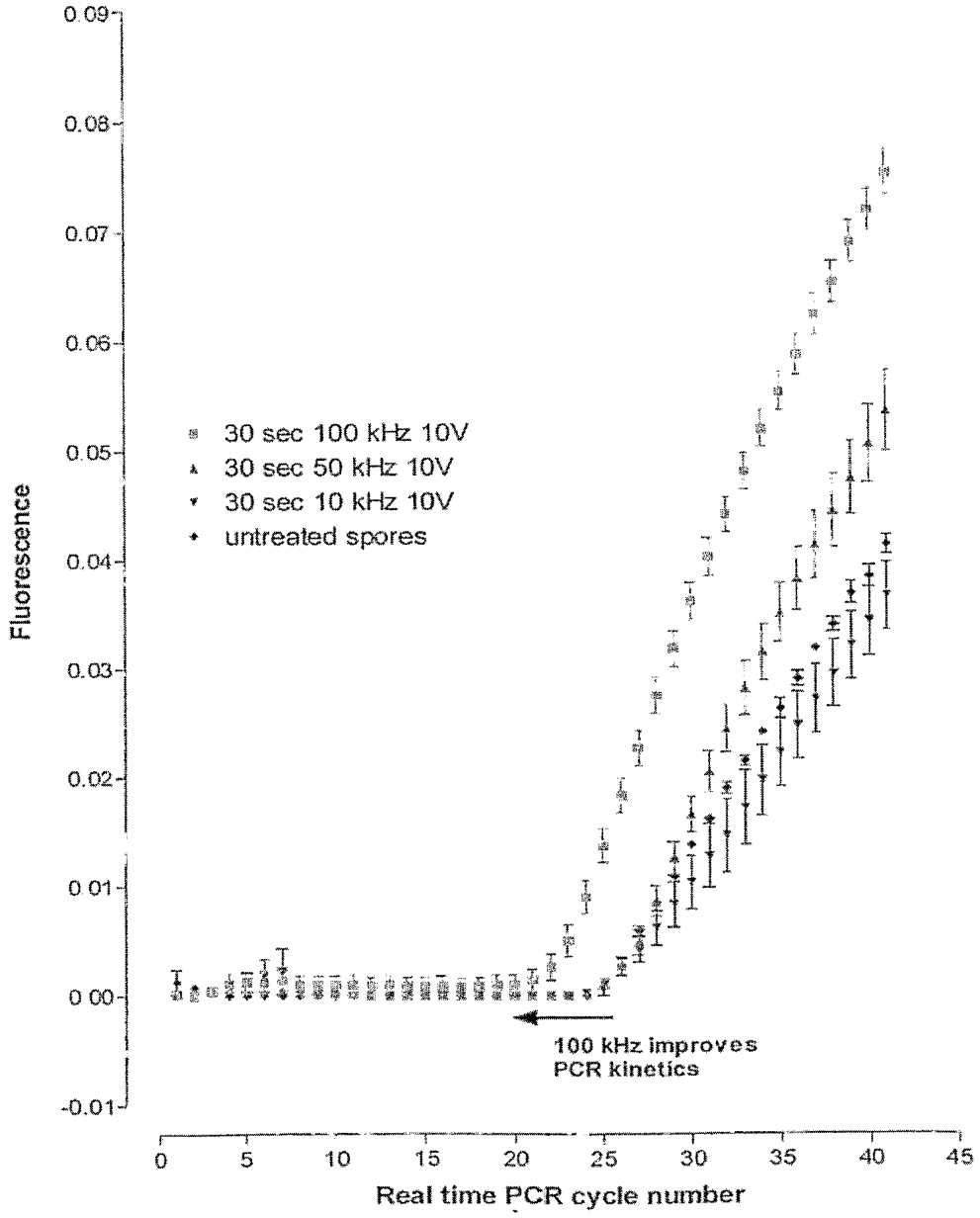

**Figure 5**

Electrolysis of spores is dependent on interfacial electrode resistance